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ROLE OF BIOFILMS IN *STAPHYLOCOCCUS* COLONISING INTRAVENOUS CATHETERS

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ABSTRACT

Introduction: *Staphylococci* are major nosocomial pathogen associated with indwelling medical devices, especially with intravascular catheter (I V) related infections. Major virulence factor of *Staphylococcus* is its ability to form biofilm on polymeric surfaces.

Objective:

- To isolate and identify the pathogens colonising IV catheters.
- To select the *Staphylococcus species* and to detect their ability to produce biofilms along with antimicrobial susceptibility testing.

Material and methods: A total of 373 IV catheter tips from 373 patients were collected and processed for isolation of bacterial pathogen. Total 119(31.9%) IV catheter tips were culture positive. 100 *Staphylococcus* strains were tested for biofilm detection by Tissue culture plate (TCP) method and Tube method (TM). Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion method as per CLSI guidelines. The time frame since insertion and removal of catheter was also noted.

Results: 125 organisms were isolated from 119(31.9%) culture positive IV catheter tips. *Staphylococcus spp*.(80%) was the most common organism followed by *Enterococcus spp*(9%). 93(24.4%) catheters were colonised within one week of insertion.56% of the *Staphylococci* were *Coagulase negative staphylococci* (*CoNS)* and 44% were *S.aureus*. Of 100 *Staphylococcus spp*. 84% were biofilm producer by TCP method and 75% by Tube method. Sensitivity and specificity of TM method vis-a-vis TCP method was 89.3%and 100% respectively.

Conclusion: Majority of *Staphylococcus* isolated from IV catheters were biofilm producers. TCP method is standard method for detection of strong, moderate and weak biofilm producing strains, but TM method is technically simple. Antimicrobial resistance was significantly higher in biofilm producing *Staphylococcal species*.

Key Words: Tissue culture plate (TCP), Tube method (TM), Biofilm, Intravascular catheter (I V).

INTRODUCTION

The use of indwelling medical devices is important in treatment of critically and chronically ill patients.[1] These indwelling medical devices become the focus for infections, like intravascular catheter related infections. Use of vascular catheters has become an indispensable part of modern medicine practice. The predominant organisms isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus*.[2]

The major virulence factor for these organisms is their ability to form biofilm.[2] Biofilm consist of slime which is a complex extracellular polymeric substance produced by most of the *Staphylococcus*.[3]

In biofilm, specific initial adherence to device surface is mediated by polysaccharide adhesion (PSA). Following initial adhesion, polysaccharide intercellular adhesion (PIA) is involved in cell-cell adhesion. The synthesis of PIA is mediated by the chromosomal *ica* gene (intercellular adhesion), which is an operon structure contains the *icaADBC* genes.[4,5]

Biofilm formation is a major concern in nosocomial infections because it protects microorganisms from host immune response along with antimicrobial agents. Such infections

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are resistant to systemic antibiotic therapy and removal of infected device becomes necessary. Therefore, once biofilm-associated *Staphylococcal* infections occur, they are difficult to eradicate.[6]

Biofilm formation in *Staphylococcus* can be studied by various methods such as-microscopic examination by using epifluroscence, scanning electron microscope (SEM), confocal laser scanning microscope (CLSM). Molecular techniques such as Polymerase chain reaction (PCR) which amplifies the gene (*ica* ABCD).[7]

The present study was carried out to assess the incidence of *Staphylococcus spp*. colonising intravenous catheters. An effort was also made to study the presence of biofilm in these *Staphylococcus spp* and their antimicrobial sensitivity pattern.

MATERIAL AND METHODS:

Collection and processing of IV catheter tips:

A total of 373 IV catheter tips (336 peripheral venous catheter tips, and 37 central venous catheter tips) were collected from 373 patients, admitted in Medicine, Surgery, Paediatrics wards, and Intensive care units. The distal 5 cm of catheter was cut off and placed in a sterile screw caped container with 1ml brain heart infusion (BHI) broth.[10] [fig.1]

All tips were processed for quantitative culture technique. For this, catheter tip in BHI broth was vortexes for 1min and 0.1 ml of suspension was spread over Blood agar and MacConkey agar [11,12]. The plates were incubated aerobically overnight at 37^0C and were observed for growth. Catheter tip colonization was said to be present if more than 100 cfu /catheter segment by this vortexing techniques. [11] A total of 100 *Staphylococcus spp*. were isolated from 373 catheter tips. Speciation was done by Standard microbiological techniques. [11] All *Staphylococcus* isolates were subjected to antimicrobial susceptibility testing on Muller-Hinton agar by Kirby-Bauer disc diffusion method[14]. Methicillin resistance was detected by using Cefoxitin-30µg disc as per CLSI.[14]Inducible Clindamycin resistance were detected by D test as per CLSI guidelines. [14]A record was kept of the time since insertion of all catheters removed and cultured in the present study.

Biofilm detection methods:

Biofilm formation in *Staphylococcus spp.* was done by tube method(TM) and tissue culture method (TCP).This was done to evaluate the usefulness of the tube method for biofilm detection.

Tube method(TM):[1,3]

10 ml Trypticase soya broth (TSB) with 1% glucose was taken in a new glass test tube which was inoculated with loopful of Staphylococcus growth from overnight culture plate. It was further incubated at 37°C for 24 hours. Tube was decanted and washed with PBS (PH 7.3) and then further it was air dried. A dried tube was stained with 0.1% crystal violet. Excess stain was removed by distilled water. Tubes were dried in inverted position and observed visually for biofilm formation. Each test was performed in triplicate to minimise errors. S. epidermidis A

TCC 35984 (strong biofilm producer) used as positive control while, uninoculated TSB broth with 1% glucose as negative Control.

Biofilm formation is said to be positive when a visible thin film lined the wall and bottom of the test tube. Ring formation at the liquid interface is not considered as an indication of biofilm formation. Biofilm formation was scored as-Strong, moderate, weak, and negative. [fig.3]

Tissue culture plate method (TCP): [1,9,15]

A. Biofilm Cultivation - All *Staphylococcal* isolates from fresh agar plates were inoculated in TSB with 1% glucose and incubated for 18 hr at 37^oC in stationary condition and then diluted 1 in100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates were filled with 200µL aliquots of the diluted cultures. The inoculated plate was covered with a lid and incubated aerobically for 24 hr at 37^0C under static condition.

B. Washing - After incubation content of each well was gently removed by tapping the plates. Wells were washed three times with 300 µL of sterile phosphate-buffered saline (PBS; PH 7.2).

C. Fixation - After washing remaining attached bacteria were fixed by air drying.

D. Staining - Adherent biofilm layer formed in each microtiter plate well was stained with 150 µL of 0.1% Crystal violet for 15 min at room temperature.

After staining, washing was done until washing are free of stain. Then microtiter plate was air dried at room temperature. *S. epidermidis* ATCC 35984 (strong biofilm producer) used as a positive control while uninoculated TSB broth with 1% glucose as negative control.

E. Measurement and interpretation of results[15]

Uniformly Stained adherent Staphylococcal cells on well of microtiter plate were considered as biofilm producer [fig.3].

Optical density (OD) of the stained adhesive Staphylococcus bacteria was determined with a micro-ELISA reader at a wavelength of 570 nm (OD570 nm). The result of the test is recorded and these OD values are considered as a true indication of bacteria adhering to the surface and biofilm formation. Tests were performed in triplicate to minimise errors & for analysis of data.

Interpretation of obtained results requires definition of the cut –off value that separates biofilm producing from non-biofilm producing strain.

i. Average OD values were calculated for all tested strain (ODt) and negative controls (since all tests are performed in three times).
ii. Cut-off value (ODc) was calculated. It is defined "as three standard deviations (SD) above the mean OD of negative value".
[ODc = average OD of negative control + (3xSD of negative control)].
iii. ODc value was calculated for each microtiter plate separately.

For easier interpretation of results, strains were divided in to the following categories.

- No biofilm producer = ODt ≤ ODc
- Weak biofilm producer = ODc < ODt ≤ 2xODc
- Moderate biofilm producer = 2xODc < ODt ≤ 4x ODc
- Strong biofilm producer = 4x ODc < ODt

Statistical Methods:

Statistical evaluation of the TM method for detection of biofilm formation-

Results and observations were analysed by using In Silico statistical software. Chi-square test and P-value was calculated by using this software for comparison and to determine the statistical significance

Results and observations:

Total 373 IV catheter tips were processed out of which 119 (31.9%) were culture positive, which include 95(28.3%) peripheral and 24(64.9%) central IV catheter tips.[Table1] Common indication for peripheral IV line removal [85 i.e.89.5%] was when no longer required. In central IV line it was removed in majority of patients [20 i.e.83.4%] due to local sign and symptoms of catheter related infection.

Peripheral IV lines were colonised with bacteria earlier than central IV line.55 (57.9%) peripheral IV catheter colonization was seen after 2-4 days of catheter use while100% colonisation with CVC when duration of catheter use was > 7days [Table 2]

Total 125 organisms were isolated from 119 culture positive IV catheter tips. *Staphylococcus spp*.100 (80%) was the most common organism followed by *Enterococcus spp* 9%.[Table 3] 6 catheter tips showed mix growth i.e. two organism from each.

Out of 100 *Staphylococcus species,* 44% were *S.aureus* and 56% *CoNS*. Amongst the all *CoNS*, *S.epidermidis* 22 (39.28%) was the commonest isolate followed by *S.haemolyticus* 13 (23.1%). [Table 4]

In present study 84% of *Staphylococci* were biofilm producer by tissue culture plate method and 75% by tube method. TCP method is considered standard test for biofilm detection. The tube method is relatively and technically simple method. Out of these 84% of *Staphylococci* 39(46.4%) were *S.aureus* and 45(53.3%) were *CoNS*. However in *CoNS* 21(52.5%) were *S.epidermidis* and 10(25 %) *S.haemolyticus*.

Sensitivity and specificity of TM method vis-a-vis TCP method was 89.3%and 100% respectively with 100% Positive predictive value (PPV), and 64% negative predictive value (NPV). Biofilm formation by tube method was found to be equivalent in specificity to the tissue culture plate method for detecting biofilms. [Table 5]

TM method was comparable with TCP method to differentiate strong and moderate biofilm producing strains but not the weak one. The TCP method was definitely better in detecting weak biofilm producer. [Table 6]

In this study >50% *Staphylococcal* spp. were multi drug resistant (MDR) [Table-7]. Overall 75% of *S. aureus and* 60.7% *CoNS* were resistant to Penicillin and Methicillin resistance was 63.6% in *S. aureus* and 42.8% in *CoNS* [Table7]. However, inducible Clindamycin resistance (MLSBi) was 21% amongst *all Staphylococcus species* and constitutive Clindamycin resistance (MLSBc) was 35%. Antimicrobial resistance was higher in biofilm producing Staphyloccus *spp*. than non biofilm forming one [Table 8].

DISCUSSION

The extensive use of intravenous catheters in hospitalized patient has led to increased incidence of catheter-related infection (CRI), especially blood stream infections. These infections originate from the microbial colonisation of the intravascular catheters. [16]

In the present study out of 373 IV catheter tips processed, 119 (31.9%) were culture positive [Table 1]. Rao SD et al (2005) reported 74(54.8%) culture positive out of total 135 IV catheter tips. This was higher than present study. This could be probably due to the fact that IV catheter tips were collected from paediatric intensive care unit (PICU) only in theirstudy.[17]

Peripheral IV catheter tip culture positivity rate, in our study was 95 (28.3%). In Nahirya P et al (2008) showed 20.7%

culture positivity of peripheral IV catheter tips collected from paediatric wards.[18]However, Rao SD et al (2005) detected 54(52.4%) were culture positive IV tips out of 103 peripheral IV catheters.[17]

Central IV catheter (CVC) tip culture positivity rate in present study was 24 (64.9%) This was comparable with 62.5% each given by Subba Rao SD et al in (2005)[17] and Gahlot R et al in (2013).[19]While Chopdekar K et al(2011) found that culture positivity rate was 57.6% in their study. [20]

Peripheral IV catheters were removed in 89.5% of patients when they were no longer required. However, Central IV catheter removal in 83.4% of patients was due to the presence of clinical sign and symptoms of infections like local signs of inflammation .Majority of CVC catheter tips were collected from adult ICUs with co-morbid conditions like hypertension, diabetes, malignancy or receiving chemotherapy.

The duration of IV catheterisation is a significant factor which determined the development of catheter related infections. In our study peripheral IV catheter colonisation was seen, in 57.9% of patients, after 2-4 days of catheter use [Table2]. These findings were comparable with Rao SD et al[17] (2005). In present study central IV catheters culture positivity rate was 100% when duration of use was more than7days [Table2]. Rao SD et al (2005) showed 100% culture positive rate, after duration more than 11days. [17]

Total 125 organisms were isolated from 119 cultures positive catheter tips in our study. Out of which *CoNS* 44(44.8%) and *S.aureus* 56(35.2%) were predominant ones, followed by *Enterococcus spp*.(9%)[Table 3]. Nahirya et al (2008) found *S.aureus* (60.5%) as the predominant pathogen followed by *S.epidermidis* (23.4%).[18] Rao SD et al (2005)[17] detected *CoNS* (32.4%) followed by *Pseudomonas spp*.(31%).While in Chopdekar et al, (2011) study revealed maximum colonisation with non-albicans Candida spp.(22.6%).[20]The microbial profiles of catheter colonisation vary in different settings or areas due to the impact of environmental contaminants in the pathogenesis of device related infections.

CoNS is frequently responsible for catheter colonization due to its capacity to adhere to polymer surfaces and consequent biofilm production. Out of 100 *Staphylococcus* spp. isolated in our study, 56% were *CoNS* and 44% were *S.aureus*. Two recent studies Prasad S et al[21] (2012) and Patil HV et al (2011) isolated 57.1% and 65% of *CoNS* respectively from indwelling IV catheter tips, which were comparable with our study.[22] Amongst the 56% *CoNS* in present study 39.28% were *S.epidermidis* followed by *S.hemolyticus* 23.1% [Table 4]. Patil HV et al (2011)[22] showed 45% of *S.epidermidis* and 15% of *S.hemolyticus* in their study.

S.aureus is a known pathogen in hospital infections. It was the second common organism in our study which was (35.2%). Rate of *S.aureus* was much lower in Khanna et al (2013) [23] who got 13.25% as compared to our study The higher rate of *S.aureus* in present study could be due to the lack of dedicated IV catheter insertion team, as well as lack of standardized protocol for insertion and replacement of IV catheters.

In the present study 100 *Staphylococcus spp.* were screened for biofilm detection by modified TCP method and TM method, along with antimicrobial susceptibility pattern.

84% and 75% of *Staphylococcus spp*. showed biofilm production by TCP and TM method respectively [Table 5]. Bose et al (2009) reported 54.2% and 42.4% of *Staphylococcus spp.* were biofilm producer by TCP and TM method respectively.[24] While Mathur et al (2006) showed 53.9% and 41.4% of *Staphylococcus spp.* as biofilm producer by TCP and TM method respectively.[1] Biofilm production in present study was higher than these studies. These could be because the isolates from above two studies were obtained from all clinical samples and not only catheter tips like the current study.

In this study 84% *Staphylococcus species* were true biofilm producer, i.e. positive by standard TCP method [Table-5]. However, 9 strains were positive by TCP but negative by TM method. This is because it was difficult to differentiate between weak and non-biofilm producers by TM method. Otherwise TM method correlated well with TCP method for Strong and moderate biofilm production.[Table-6]. TCP method was definitely better test to detect weak biofilm producing strains. This variability in results of weak biofilm production was also observed by other similar studies.[1,24]

Sensitivity and specificity of TM method vis-a-vis TCP method was 89.3% and 100% respectively with 100% Positive predictive value (PPV), and 64% negative predictive value (NPV) [Table-5]. This finding is supported by two similar studies. [1,24]

Multidrug resistance (MDR) was significantly higher in all biofilm producing strains than non biofilm producing strains in present study [Table-8]. MDR strain was taken as a strain resistant to > 2 classes of antibiotic. Thus making it difficult to treat intravenous catheter related infections.

There are some highly accurate methods like PCR to detect *ica*ADBC operon. These encode polysaccharide intercellular adhesion (PIA) which mediates biofilm formation. These are expensive methods. In a developing country like India, low cost method like TCP and TM methods are useful for screening purpose of biofilm producing strains.

CONCLUSION

To conclude, present study showed biofilm formation in 84% of the *Staphylococcus species* isolated from IV catheters tips.

Since this colonisation will result in blood stream infections unless the catheter is removed or changed early. Simple preventive measures, such as aseptic precaution during catheter insertion, daily catheter care, monitoring of catheterised patients, could help to reduce risk of colonisation and subsequent catheter related infections. Since these infections are difficult to treat, it is better to prevent such infections than attempt to treat, once they are established.

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Table 1: Distribution of total culture positive catheter tips (no. =119)

Catheter tips	Culture positive
P.IVcath tip (336)	95 (28.3 %)
C.IVcath tip (37)	24 (64.9%)
Total	119 (31.9%)

Table 2: Correlation between duration of IV catheterisation and catheter culture positivity.

Days of Catheterisation	Peripheral I.V line	Central I.V line
2-4	55 (57.9%)	-
5-7	38 (40%)	-
8-10	2(2.1%)	14 (58.3%)
11-13	-	7(29.2%)
14-16	-	3(12.5%)
More than 17	-	
Total	95	24

Table 3: Distribution of 125 organisms from 119 culture positive IV catheters tips.

Organisms	Total
CoNS	56 (44.8%)
S.aureus	44 (35.2%)
Enterococci	11 (9%)
Pseudomonas.spp	8 (6%)
Candida spp.	4 (3%)
E.coli	2 (2%)
Total	125

Table 4: Distribution of Staphylococcus spp. isolated from I.V catheter tips.

Staphylococcal spp.	Total no.(n=100) No
1. *CoNS*	56
S.epidermidis	22 (39.28%)
S. haemolyticus	13 (23.1%)
S. warneri	3 (5.34%)
S. cohnii	3 (5.34%)
S. hominis	3 (5.34%)
S. sciuri	2 (3.6%)
S. caprae	2 (3.6%)
S. saprophyticus	2 (3.6%)
S. auricularis	2 (3.6%)
S. xylosus	2 (3.6%)
S. hyicus	2 (3.6%)
2. *S.aureus*	44
Total	100

Table 5: Evaluation of Tube method(TM) Vis-a-vis Tissue culture plate (TCP) method in biofilm formation.

Method	TCP positive	TCP negative	Total	X2 value	P value
TM positive	75	0	75	57.14	< 0.0001 (Statistically significant)
TM negative	9	16	25		
Total	84	16	100		

Sensitivity	Specificity	PPV	NPV
89.3%	100%	100%	64%

Table 6: Biofilm formation in *Staphylococci* by TCP and TM method

	Tissue culture plate method (TCP)	Tube method (TM)
Strong biofilm	26 (30.9%)	25 (33.3%)
Moderate biofilm	28 (33.4%)	26 (34.7%)
Weak biofilm	30 (35.7%)	24 (32%)
Total (n=100)	84 (100%)	75 (100%)

Table 7: Antimicrobial resistance pattern in Staphylococcus spp.

Antibiotics	*S.aureus* (n=44)	CoNS (n=56)	Total (n=100)
Penicillin	33 (75%)	34 (60.7%)	67
Gentamicin	23 (52.3%)	31 (55.3%)	54
Ciprofloxacin	20 (45.4%)	28 (50%)	48
Cotrimoxazole	27 (61.4%)	26 (46.4%)	53
Tetracycline	27 (61.4%)	23 (41%)	50
Erythromycin	25 (56.8%)	24 (42.8%)	49
Clindamycin	16 (36.4%)	19 (33.9%)	35
Linezolid	0	0	0

Table 8: Evaluation of MDR strains in biofilm forming and non biofilm forming Staphylococcus spp.

	Biofilm forming strains	Non biofilm forming strains	Total	Chi-square test	P value
Staph.spp.	84	16	100	4.87	0.0272 (Statistically significant)
MDR	61	3	64		

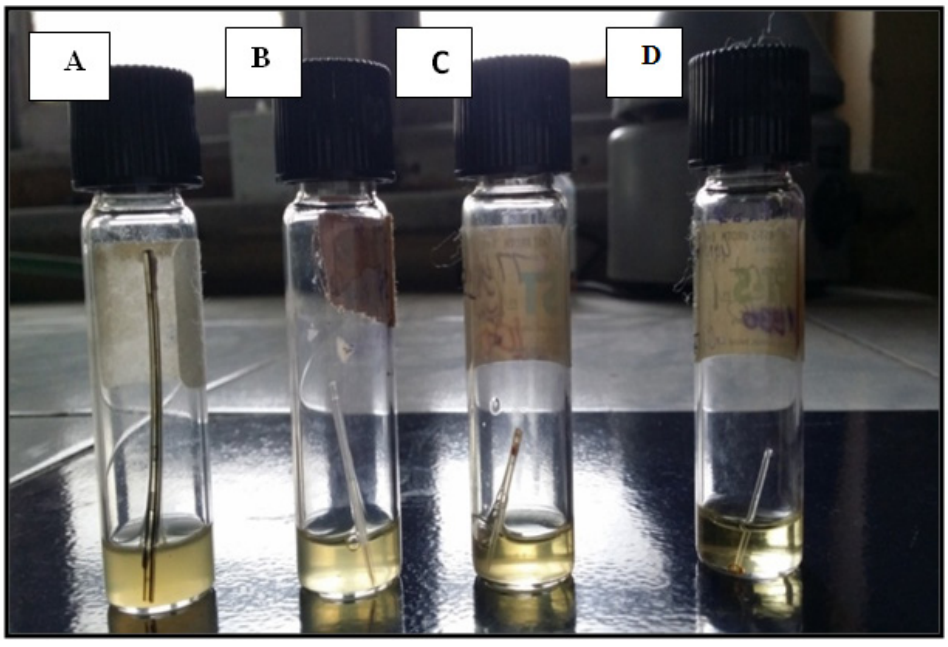

Figure 1: Collection of Intravenous catheter tips
A - Central IV catheter tip.
B,C,D- Peripheral IV catheter tip

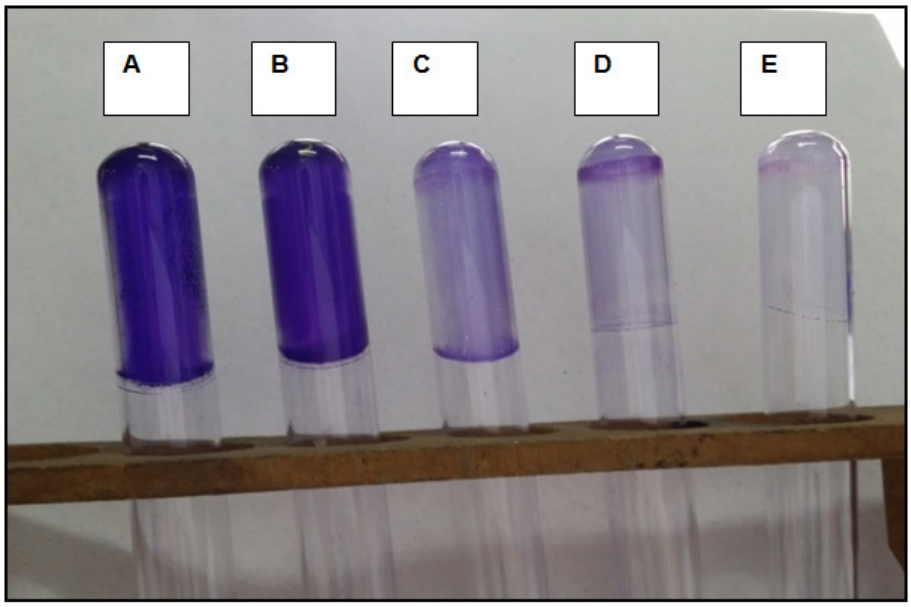

Figure 2: Biofilm detection in *Staphylococci* by Tube method (TM) method
A. PC- positive control
B. Strong- Strong biofilm producer
C. Moderate- Moderate biofilm producer
D. Weak-Weak Biofilm producer
E. NC- Negative control

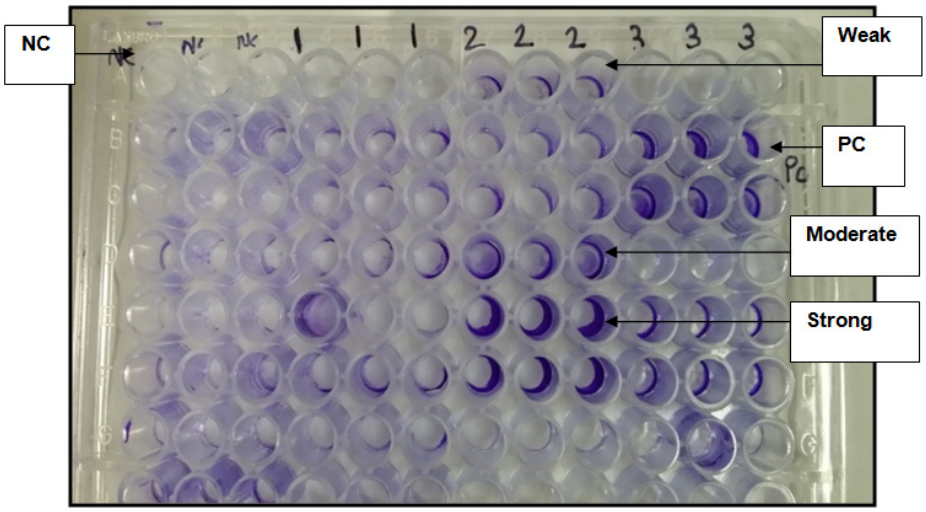

Figure 3: Biofilm detection in Staphylococci by Tissue culture plate (TCP) method
PC- Positive control
NC- Negative control
Strong- Strong biofilm producer
Moderate- Moderate biofilm producer
Weak-Weak biofilm producer